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Properties of a Water-Soluble Paclitaxel Conjugate in Aqueous Solution and its Interaction with Serum Albumin

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Summary: A summary of the most recent investigations of conformation and solution properties of a polymer conjugate of the anti-cancer drug paclitaxel is given. First results of spectroscopic studies of interaction with model proteins in the solid state and in solution are discussed.

Keywords: albumin; interaction; paclitaxel; solution properties

Introduction

The efficacy of pharmaceutical drugs depends strongly on their solubility, stability, and distribution in the body. These properties are not only influenced by their intrinsic solution properties but also by their specific or unspecific interaction with constituents of the body fluid such as blood or serum, and their transport properties through membranes and interaction with tissue. There are different reasons why a drug is not directly applied but a polymer conjugate is used. The reason, for example, can be the low solubility of a lipophilic drug in an aqueous medium, the need of a drug-delivery system that provides a controlled release, transport of a higher local density of the drug (for example in micelles, vesicles or nanoparticles), accumulation in tumour tissues, or protection of the drug against environmental parameters such as a certain pH – range *etc*. This type of a polymer-modified drug is also termed ''prodrug'' which means a ''harmless substance which undergoes a reaction inside the body to liberate the active drug'' [1]. The term ''predrug'' is also used to indicate that the drug conjugate is not yet active, *e. g.* [2].

An enhanced vascular permeability (super-permeability) is frequently combined with an improved retention and hence the whole phenomenon is abbreviated EPR. The EPR-effect is not only observed in tumour tissue but also in inflammatory and infected area and in granuloma, see for example Maeda [3] or Peterson and Apergren [4]. As a result of EPR extravasation of lipid particles, plasma proteins and also macromolecules into the interstitial space is observed. Studies by Maeda *et al.*[5–7] or [8], for example, have demonstrated such an accumulation of chicken ovomucoid (29 kDa) and plasmaproteins larger than 60 kDa. Therefore, the leakage of certain lipids and macromolecules into the interstitial space and their clearance via the lymphatic system can provide an excellent lymphatic delivery system that may help to prevent lymphatic metastasis. It is in particular helpful for the accumulation in tumours that the lymphatic clearance rate out of tumour tissue is markedly slower than from inflammatory tissue. This is in particular true for macromolecular substances where the retention in tumours is significantly higher compared with low molar mass

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substances. Diffusion of small molecules back into the blood-circulating system is much faster compared with macromolecular substances [7].

Apparently, self-aggregation and complex formation with constituents of body liquids are of vital importance for the fate and the bioavailability of drugs. In blood there is a competition for docking sites on transport proteins between many substances. The most important transport protein is albumin. Human serum albumin (HSA), for example, has an abundance of 5 g/cL blood and a high affinity to a wide range of substances such as bilirubin, warfarin, amino acids, fatty acids, the anticancer drug paclitaxel (generic name), even to 2-acetoxybenzoic acid (Aspirin®) or Cu^{2+}- and Zn^{2+}-ions. A novel receptor mediated albumin-bound paclitaxel transport mechanism has recently been described on a conference [9]. This competition for the docking sites on transport proteins is in particular important in combined therapies or when a drug blocks a docking site that is otherwise responsible for the removal of metabolites such as bilirubin.

Paclitaxel (or taxol*) – M = 853.92 g/mol – is a pharmaceutical active substance of the taxol family that can be extracted from the bark of the Asian yew tree (*taxus brevifolia*) or other taxiceae. This tetracyclic taxan-type diterpen, Fig. 1 and 2, is lipophilic with only a poor water solubility (≈0.6 mmol/L) [10]. Farina reports a solubility in water <0.01 mg/mL [11]. The different solubility values are probably due to the existence of less soluble hydrates or differences of the solubility of crystalline respectively amorphous paclitaxel [12].

Paclitaxel is well-known for its anticancer activity against a number of different types of cancer such as breast-, lung- or ovary cancer [13]. In recent years it also drew attention in fighting against restenosis after percutan transluminal angioplasty. Paclitaxel interferes in cell replication by

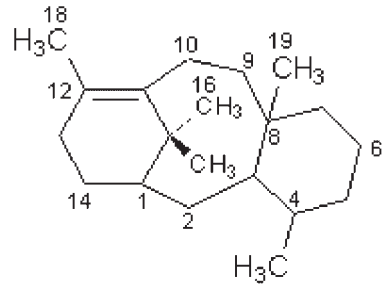


Figure 1.
Diterpenoid taxane structure.

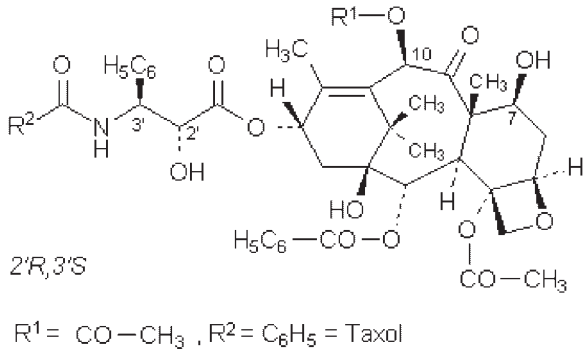
stabilising the microtubuli in the late G_2 phase of the mitosis so that the cells are unable to assemble the mitotic spindle [14], see Fig. 3. The cytotoxic activity of paclitaxel requires an intact taxane body and an ester side chain at the carbon C-13. Modification of the oxetane ring, the benzyloxycarbonyl at C-2, the acetoxy group at C-4 and the phenyl group at C-3′ decreases significantly the bioactivity. Also, the hydroxyl group at C-2′ should be free accessible for significant bioactivity.

Oral administration of the drug is problematic because it has a poor absorption due to the poor water-solubility, and also the efflux pump function of the drug for the multi-drug transporter P-glycoprotein (Pgp) (which is abundant in the gastrointestinal tract) is poor. Consequently, paclitaxel is mainly used in intravenous administration [15] but still the problem of the poor solubility prevails.

In order to apply the drug directly and fast to the body, paclitaxel has to be hydrophilised to make it soluble in blood plasma. Dissolving aids such as poly(oxyethyleneglycerol) trioleates or Cremophores® can cause undesired side effects [16]. Cremophores® are non-ionic emulgators consisting of ethoxylates of fatty alcohols respectively hydrated Castor oil or nonylphenol.

An alternative way is to couple paclitaxel *e. g.* through an ester linkage at the carbons 2′ or 7 (see Fig. 2) with a succinic acid functionalised poly(ethylene glycol) (PEOS) [17,18].

*Taxol® is also a registered trade name of Bristol-Myers-Squibb, New York, N. Y.



$R^1 = CO-CH_3$, $R^2 = C_6H_5$ = Taxol

Figure 2.
Paclitaxel (Taxol) M = 853,92 g/mol

To become fully active, the hydrophilic substituents have to be removed from the drug by enzymes such as an esterase. The first derivatives, however, were stable for several hours in plasma liquid so that most of the prodrug was excreted before it could become active.

This problem was overcome with the development of the prodrug by Jo [19] who connected the hydrophilic polymer with the paclitaxel molecule at the carbon 7 or carbon 2′ with a self-immolating linker, see Fig. 4. In the following we report about a polymer conjugate coupled with paclitaxel at C-7, which is therefore termed PP7. In contrast to earlier water-soluble paclitaxel derivatives PP7 is much more rapidly hydrolysed by body-intrinsic esterases.

With the self-immolating linker a reduction of the half-life time of the prodrug could be achieved from several hours down to less than 10 minutes (in rat plasma). This increases the bioavailability drastically. The basic principle of the polymer-drug conjugate is represented by the following structure that also shows the general

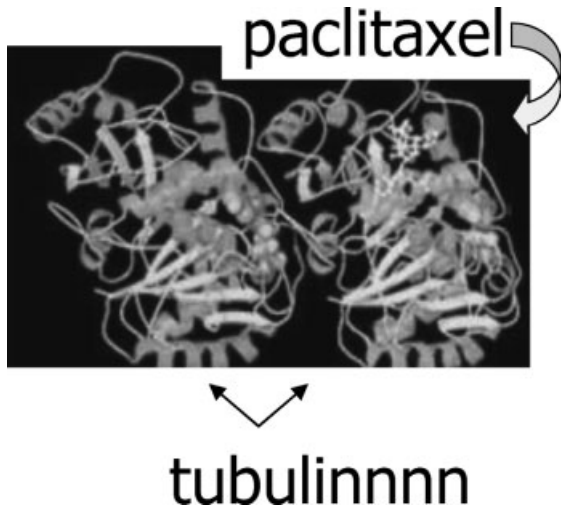


Figure 3.
paclitaxel stabilising a tubulin dimer, modified from M. Malaloom, D. Crètien, E. Karsenti, J. H. K. Hørbe, *J. Cell Sci.***1994**, *107*, 3127-3131.

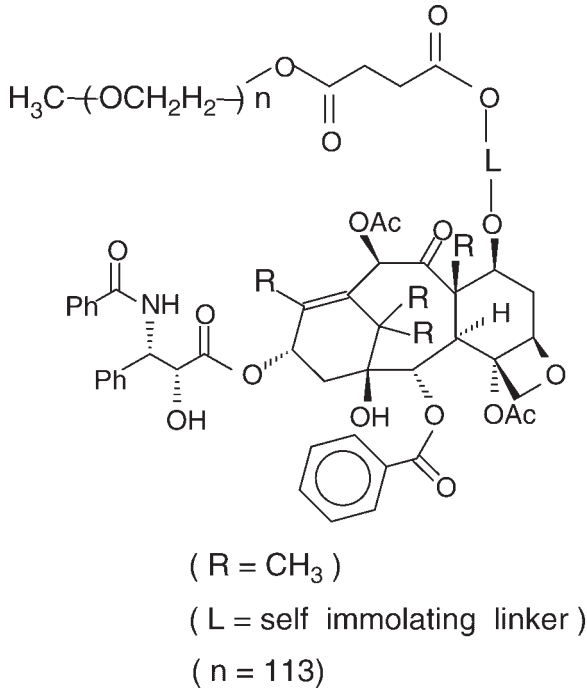
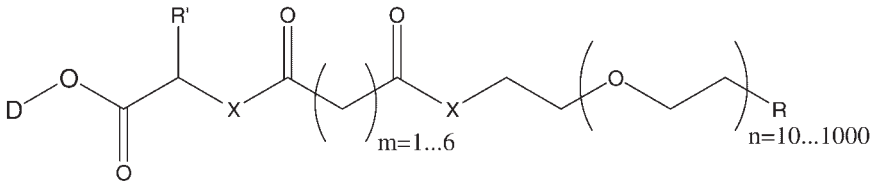
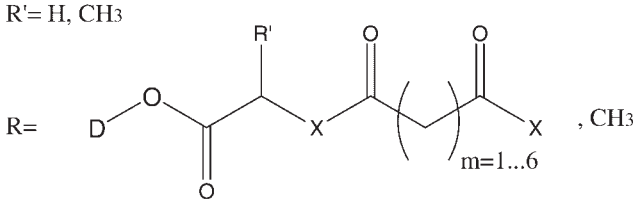


Figure 4.
in the prodrug PP7 paclitaxel is connected with the water-soluble poly(ethylene glycol) chain through a self-immolating linker [19] on C-7.

structure of the self-immolating linker L in Fig. 4[19]:



D = paclitaxel or derivative thereof
X = O, S, NH
R'= H, CH3



Because of the comparably low stability of PP7 in serum liquid it is important to have information about interactions and mobility of the molecules in aqueous solutions. In particular it is important to study concentration-depending behaviour with respect to the possible formation of molecular aggregates. In a series of previous papers we

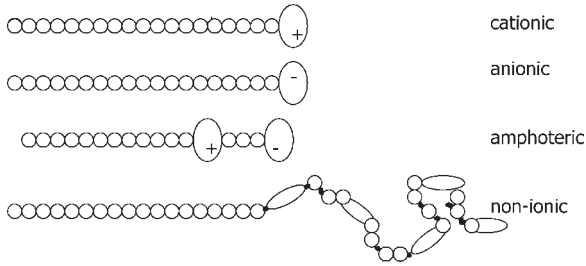


Figure 5.
classical tensid molecules.

investigated homo-interactions and properties of PP7 in aqueous solution [20–25].

Properties of Hydrophilised Paclitaxel in Aqueous Solution

The paclitaxel-poly(ethylene glycol) conjugate PP7, see Fig. 4, consists of a hydrophilic methyloxy-terminated mPEG chain of about 114 repeating units (corresponding to the maximum of the distribution curve) and the lipophilic drug. The structure resembles formally other amphiphilic molecules that tend to form associates in solution like tensids or lyotropic substances, see Fig. 5 and 6. Formation of supramolecular structures like micelles would be welcome since they provide a higher local concentration of the drug combined with the EPR-effect mentioned above. The synthesis of micelles prepared from blockcopolymers filled with paclitaxel was described Burt *et al.*[26]. Closer inspection of the solution properties of PP7 in pure water as well as in modified Hank's solution (pH 7.4), however, showed no surface activity and there are no indications for the formation of superstructures in solution. Different methods were applied and they are described in detail in ref. [19–24].

The modified Hank-solution consists of NaCl (103 mmol/L), KCl (4.0 mmol/L), Na_2HPO_4 (0.157 mmol/L), KH_2PO_4 (0.33 mmol/L), and $NaHCO_3$ (2.45 mmol/L).

Fig. 7 shows how the formation of micelles is reflected in the change of different physical properties. The concentration dependence of the diffusion coefficient D and the relative viscosity increment η_i (also called specific viscosity, η_{spec}) contain also information about the shape of the molecules in solution.

No critical micelle concentration (cmc), Figs. 8–10, was observed, and the behaviour of the conjugate PP7 with respect to the surface tension is very similar to solutions of pure PEG. That means that the

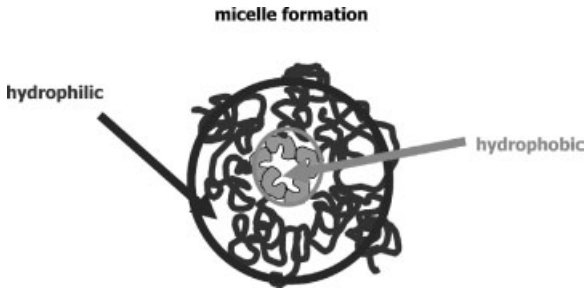


Figure 6.
micelle formed by tensid molecules comprising of a flexible, coiled hydrophilic chain and a rigid hydrophobic part.

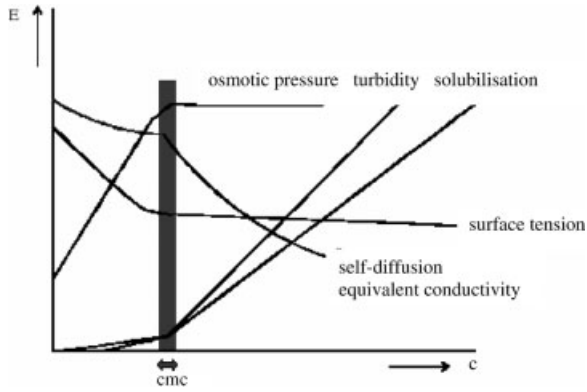


Figure 7.
physical properties E (examples as indicated in the figure) and critical micelle concentration (cmc).

PP7-molecules in solution cannot have the typical structure of a classical surfactant (tensid) as shown in Fig. 5, respectively its inversion comprising of a hydrophobic head (paclitaxel) with a hydrophilic tail stretching away from the hydrophobic part.

The concentration-dependence of the diffusion coefficient D and the relative viscosity increment η_i are sensitive to the shape of the molecules in solution, in particular concerning deviations from the behaviour of solid, non-draining hard spheres. The Stokes-Einstein equation for the self-diffusion of rigid spheres with the hydrodynamic radius R_H dispersed in a solvent of viscosity η yields together with the Einstein's equation the relation:

$$D = D_0(1 - \lambda \cdot \phi + \ldots) \quad (1)$$

where ϕ is the volume fraction of the polymer given by:

$$\phi = \frac{c}{c^*} \quad (2)$$

c is the mass-concentration of the polymer and c^* is the overlap-concentration. c^* is the concentration where the coils in solution start to overlap with each other. It is given

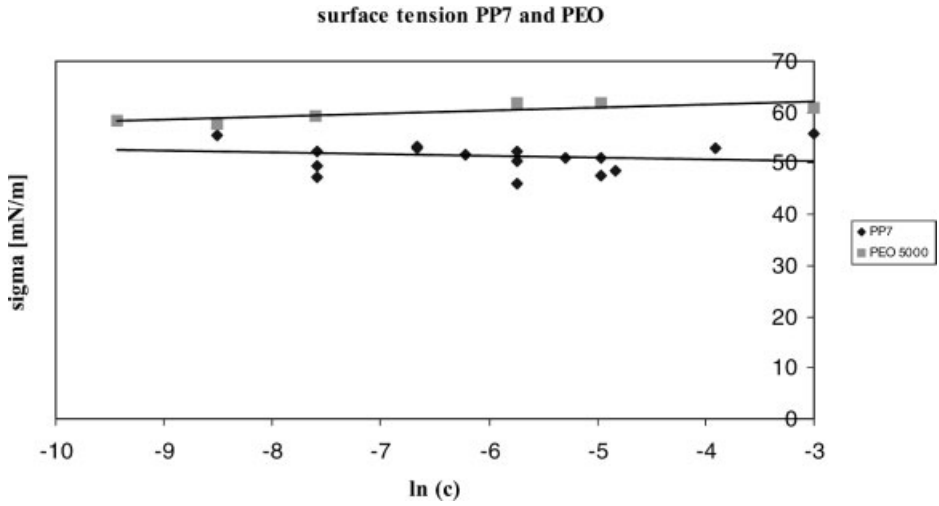


Figure 8.
surface tension vs. concentration of the polymer-drug conjugate PP7 and poly(ethylene glycol) with an average molar mass of 5000 g/mol that carries a methoxy-group on one end.

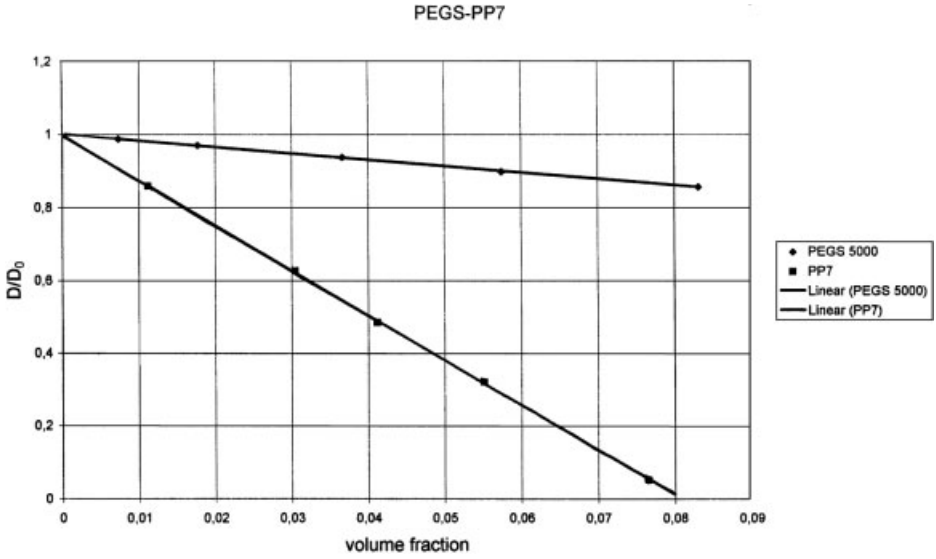


Figure 9.
Diffusion coefficient *D vs.* The volume fraction of PP7 respectively the volume fraction of a poly(ethylene glycole) that carries on one end a methoxy-group while the other end is esterified with succinic acid (PEGS). The average molar mass of the PEG-chain in both cases is 5000 g/mol, see also later.

by:

$$c^* = \frac{M}{\frac{4}{3}\pi \cdot R_H^3 \cdot N_A} \qquad (3)$$

M is the molar mass of the solute and N_A is Avogadro's constant. Although the term λ in eq. 1 is only called rigidity factor its value is also influenced by the shape of the

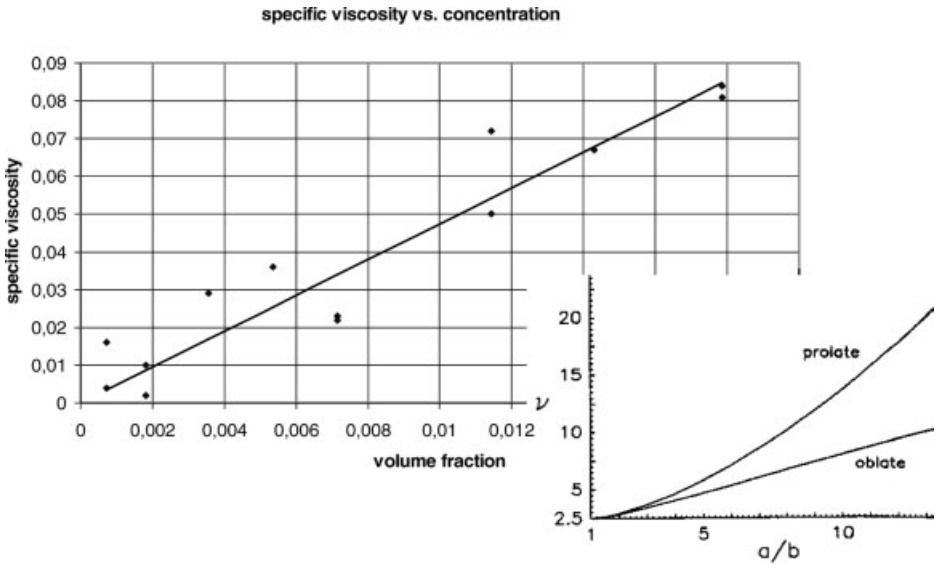


Figure 10.
solution viscosity of PP7 compared with the Simha-plot of Einstein's shape facor ν *vs.* the aspect ratio of a prolate (≈American football) respectively an oblate (discus) shaped body. For a perfectly rigid sphere $\nu = 2.5$.

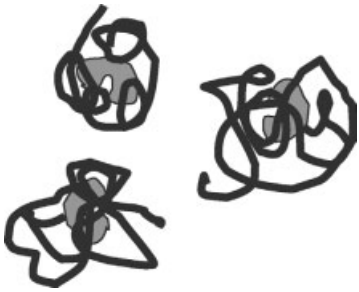


Figure 11.
model of the conformation of individual PP7-molecules. The PEG-chains provide a hydrophilic surface and the paclitaxel moieties are isolated from one another.

molecule. $\lambda = 2$ in case of a rigid, non-draining hard sphere. For details see for example ref. [27–31] and the literature quoted therein.

Comparable information is available from measurements of the solution viscosity and examination of the Einstein-coefficient ν in eq. 4 [32]:

$$\eta_{spec} = \nu \cdot \phi \tag{4}$$

For non-draining hard spheres $\nu = 2.5$. The equation has been extended by Simha [33] for deformed spheres. Molecular dynamics calculations have revealed a highly flexible PEG-chain that tends to coil around the paclitaxel molecule so that in particular in aqueous solution the hydrophobic drug is ''wrapped up'' by the hydrophilic PEG, Fig. 11. This may be compared with the hydrophobic interaction that is observed in the coiling of proteins, see for example ref. [34]. This model is supported by the fact that the phase diagram of the system PP7/H_2O is very similar to the phase diagram of PEG/H_2O [25]. No phase transitions corresponding to paclitaxel were recorded, only those of the polymer. The PEG-chain in PP7(5000) behaves like an unmodified PEG(1000)-chain. The number in parenthesis refers to the (average) molar mass of the PEG-chains.

The summary of the results given above display a model of PP7 consisting of a core-shell-molecule with highly mobile PEG chain coiled around the paclitaxel as roughly represented in Fig. 11. The coil appears to be slightly shrunken in diameter compared with the coil formed by unsubstituted PEG-chains under the same conditions. This effect can be explained by hydrophobic interaction [35]. Viscosity and diffusivity data indicate a significant deviation from a spherical shape of the dissolved molecules.

Interaction with Albumin

Once administered to the body any substance is faced with many substances, competing reactions and defence mechanisms present in a living biological system. We are now focussing on interactions with blood serum constituents and summarise first result in the solid state and in solution. Experimental details will be published elsewhere [36,37].

Blood contains about 65–80 g/L proteins, 60% of which is albumin the rest consists mainly of the different globulins (α_1, α_2, β, and γ). The different globulin fractions are complex mixtures and execute different transport functions (*e. g.* bilirubin or cortisone), they carry enzyme activators or inhibitors, and bind products of erythrocyte degradation. The γ-globulins play an important role in the immune response. All these constituents are candidates for drug-interactions. They can compete in interaction which is of particular interest in drug combination therapies.

About 30%...40% of the body's albumin is found in the intravascular space, the extravascular space holds about 210 g of human albumin. The main purpose of the albumin is to sustain the colloid-osmotic pressure (oncotic pressure). Another important function of albumin is the transport of lipids, pharmaceuticals [38,39], bilirubin, metal ions, hormones, vitamine B12, etc. [40]. Albumin has three binding sites: a neutral, an acidic and a basic binding site. At pH 6.4...7.4 it is strongly negatively charged [41]. It is a rather unspecific, universal carrier. This type of carrier is important in the transport of lipids and

lipid-soluble substances, Carter and Peters [36,37]. Albumin – it differs in the amino acid composition from species to species (HSA, BSA, pig-, sheep-, horse-*etc.* albumin) and has some indirect control of the bio-activity of the substances mentioned above since substances are considered inactive as long as they are bound to proteins. These binding properties are complementary to the volume-expanding properties of albumin [42] and of particular importance for drug delivery and bioavailability [9]. Albumins also stabilise enzyme solutions and can show certain enzymatic activities. Serum albumin should not be confused with ovalbumin (albumen) that has an entirely different structure and function.

Albumins interact with a great variety of substances for different purposes. Therefore, these serum proteins are of particular interest in the present context since drug-interaction competes with all these different compounds, and this can be an advantage as well as a disadvantage. Among the endogenous substances associating with albumin are fatty acids, bile acids and steroids, bilirubin – a toxic metabolid of heme inhibiting the oxidative phosphorylation, warfarin – a coumarin derivative – everal vitamins, amino acids and inorganic ions. It can act as a depot, hence increasing the bioavailability of the correspondent compound well beyond its plasma solubility. Complex formation can decrease the toxicity of a substance and decontaminate by transportation to disposal sites. The complex formation can also constrain a substrate in a certain conformation that facilitates metabolic processes. The binding constants range from about 10^2 mol^{-1} (Cl^-) over 10^7 mol^{-1} (long-chain fatty acids) to 10^{16} mol^{-1} (Cu^{2+}). The number of ligands can vary over a wide range, Peters [39], and there are different binding sites. Recently, interaction of BSA with sinapic acid – a widely used matrix substance in MALDI-TOF spectroscopy – has been proven with fluorescence spectroscopy [43] and up to 16 serapic acid ligands on BSA are assumed to form a complex equilibrium in solution.

Interaction of albumin with bilirubin or warfarin are of special importance since a strongly fluorescent complex with tryptophane (abbreviation trp or W) is formed that can be used to monitor the situation within the closer environment of the corresponding site. There are two trp in bovine serum albumin (BSA) – trp 214 (in fact 213 see below) and trp 134 – but only one in HSA – trp 214. There are sometimes differences in literature concerning the position of the amino acids. These can be caused by incorporation of sequences present in pre- or prepro-albumin. Or in BSA, for example, there is a gap at position 116 compared with HSA, and for comparison all numbers >116 should be increased by one, so that trp 213 in BSA becomes 214. There are 585 amino acids in HSA and 583 in BSA. There is also a derivative of albumin – proalbumin – with an additional hexapeptide at the N-terminal end of albumin and a precursor – preproalbumin – with a signal sequence of usually 18 additional amino acids. In literature the different types are sometimes not discriminated. An albumin with three trp is usually a preproalbumin (BSA) with 607 amino acids.

Although the shape of the different serum albumins is rather similar: like a heart with a deep hydrophobic pocket, see Fig. 12, 13, the sequence of amino acids shows a fairly large variety. The complete sequence of HSA, for example was published by Minghetti et al. [44], derived from the cDNA sequence, respectively by Holowachuk [45] for BSA. Three structural homologous, highly helical domains form the heart-shaped albumin protein, Goodsell [46].

While there are six to eight binding sites for fatty acids, rather randomly distributed over the molecule, see for example Goodsell [46] there are two major binding sites for all kinds of drugs, metabolids *etc.* according to the studies of Sudlow [47]:

Site I: in subdomain IIA, loops 4-5, lys 199 to ala 291

Site II: in subdomain IIIA, loops 7-8, pro 384 to ser 489

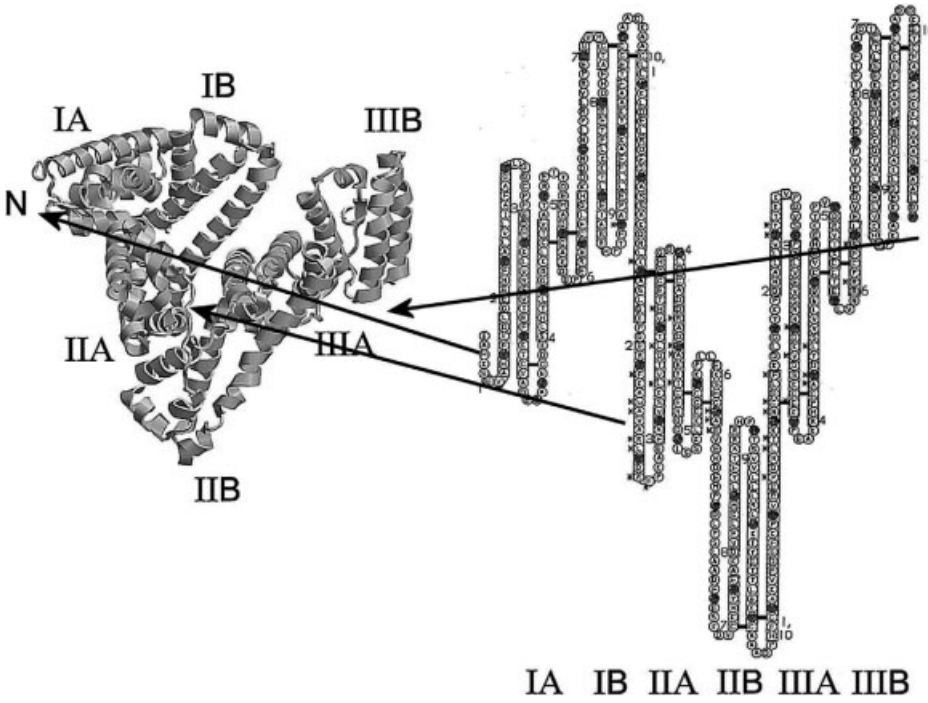


Figure 12.
HSA, amino acid sequence and identification of the subdomains after [39,47], molecular structure after [46]. Presentation with RASMOL.

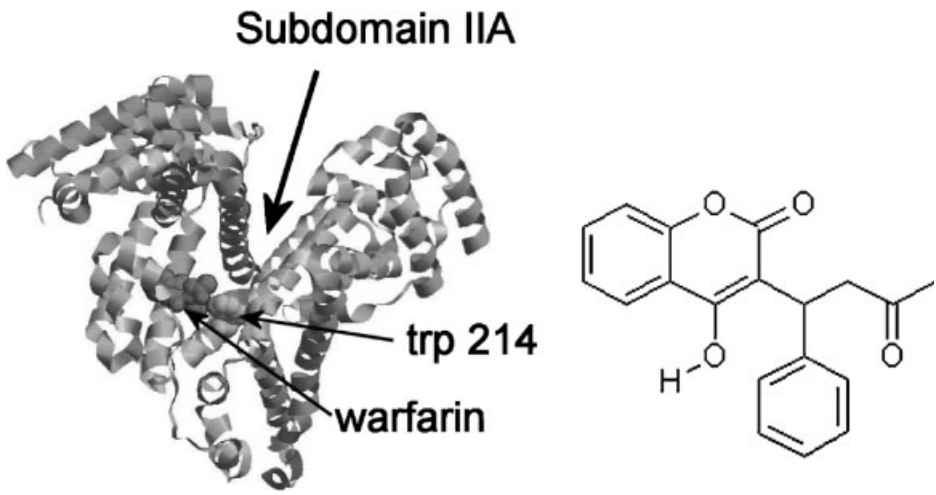


Figure 13.
subdomain IIA, fatty acid ligands, and warfarin in the vicinity of trp 214 forming a strongly fluorescent complex at 380 nm when excited at 335 nm. Presentation with RASMOL, data from the protein data bank using the entries1AO6, 1BJ5, 1BKE, 1BM0, 1E78, 1E7A, 1E7B, 1H9Z.

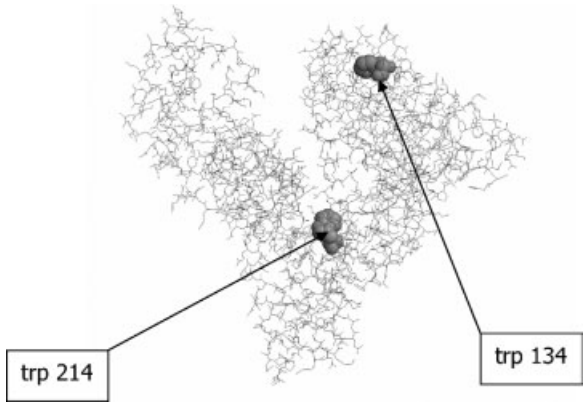


Figure 14.
BSA with the internal trp 214 in the hydrophobic drug-binding site Sudlow I and the rather surface near trp 134 in a more polar environment. Presentation RASMOL.

Site II is known to catalyze hydrolysis of some esters.

Albumins are a rather flexible molecules that can undergo rapid expansions, contractions and flexions (''breathing'') and these conformational changes are not only influenced by pH and temperature but also by ligands. It takes about 0.1 s–0.3 s to accommodate a ligand binding, Peters [39].

Many different types of ligands interact with Site I, among them many drugs, and the fact that the fluorescing trp 214 is located in this hydrophobic pocket makes this site attractive for fluorescence studies. In this context it is important that HSA has only one trp in the hydrophobic environment of the pocket buried inside the molecule, while BSA has a second trp in a more polar environment at or near the surface, trp 134, see Fig. 14.

Ligands binding to Site I are bilirubin, warfarin, salicylates, and a large number of miscellaneous compounds such as ω-dicarbonic acids of medium chain length, bulky heterocyclic anions, cyclic eicosans and hematin. Also paclitaxel appears to show an affinity for this site Trynda-Lemiesz *et al.*[48,49]. According to Fehske *et al.*[50] and Kragh-Hansen [51,52] Site I is a highly flexible region probably consisting of different overlapping sites. It is important to note that ligands binding to one site induce conformational changes in the other domain since they share an interface [53,54]. This can not only change the environment of a particular site but it can also alter the interaction with other possible ligands significantly. It can be assumed that different active principles of a multi-drug application compete for the binding sites in particular if they contain the same or similar molecule parts that are assumed to interact with the binding sites on HSA, that is the terpenoid core of paclitaxel, see Fig. 1 and Fig. 2. Poly(ethylene glycol) – PEG – is known to camouflage surfaces and to facilitate the preparation of ''stealth'' particles which are not intercepted by opsonins or detected by humoral reaction of the immune system. However, recent reports indicate interactions of the PEG with molar masses $< 10{,}000$ g/mol (which corresponds to a degree of polymerisation of about 230) with proteins, in particular with albumin [55–58]. That means that also interactions of the usually as inert regarded PEG have to be considered.

The interaction of paclitaxel and PEG can be strongly influenced when both are combined to the predrug PP7 where steric effects might prevent interaction of the terpenoid structure element of the taxol with the trp 214 binding site of HSA. However, there is still the PEG-albumin interaction that might act as suggested by Azegami *et al.*[56]. The water/PP7 phase diagram suggests that the PEG chain coils around paclitaxel, thus preventing the drug

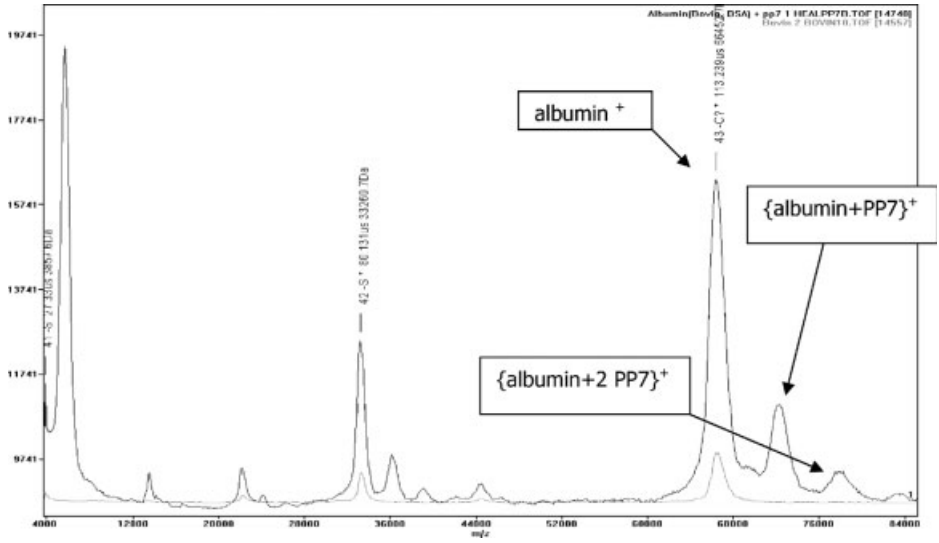


Figure 15.
MALDI-TOF of BSA (lower trace) and BSA and the PP7 adducts on the upper trace as indicated. The doubly charged ions are grouped around 33,000 Da and the triply charged ions around 22,000 Da. For details see [36].

from interaction with other paclitaxel molecules because neither the crystalline ($T_m \cong 220\,^\circ C$) nor the glassy phase ($T_g \cong 160\,^\circ C$) of paclitaxel is observed when it is bond to a PEG chain with a degree of polymerisation around 114 (equivalent to an average molar mass of 5,000 g/mol with dispersion $\langle M_w \rangle / \langle M_n \rangle = 1.05$). The melting point of PEG, however, is clearly visible [25].

We have studied the interaction of the predrug PP7† with BSA and HSA in the solid state that is formed by evaporation solvent from an aqueous co-solution and report in the following text the results of matrix assisted laser desorption ionization-time-of-flight mass spectroscopy (MALDI-TOF) experiments. First results of interaction in solution will be presented but are discussed in subsequent publications [36,37].

Interactions in the Solid Phase

MALDI-TOF spectra of mPEG (5000), the number indicates the average molar mass of a narrowly distributed mPEG, and the paclitaxel conjugate clearly shows the successful chemical modification of the polymer with the drug in that each of the individual degrees of polymerisation of PP7 shows a mass of 854 Da higher than the corresponding starting material.

When a solution of PP7, albumin and a suited matrix substance, for details see ref. [36], were evaporated and submitted to a conventional MALDI-TOF experiment it became obvious that not only the pure components PP7 (≈5800 Da) and albumin (≈66,400 Da) were undegraded transferred into the vapour phase but there were also stable aggregates of $\{albumin + 1PP7\}^+$, $\{albumin + 2PP7\}^+$, and $\{albumin + 3PP7\}^+$ + detectable, probably even higher complexes. Also, the doubly-charged ions were detectable and there were even hints of the triply-charged ion of the complexes, see Fig. 15. There is even a faint echo of the fine-structure of the PP7 chain-length distribution function in the strongest peaks of the complex ion. Fig. 16 shows the analogous experiment with BSA and mPEG (5000). There is no interaction in the solid state that prevails in the vapour phase in a MALDI-TOF experiment. The most likely interpretation presently is that

†all experiments described in the following (as before) refer to PP7(5000)

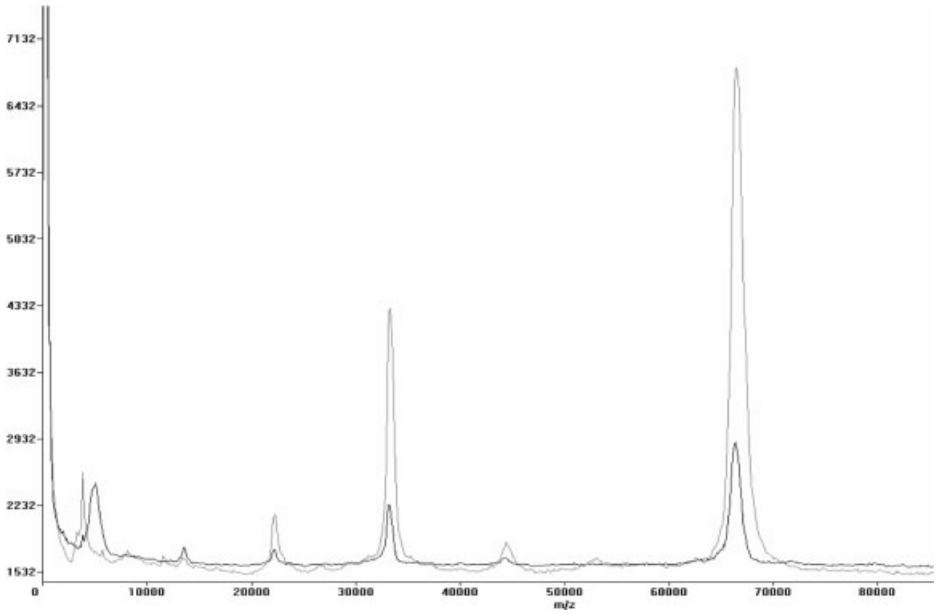


Figure 16.
MALDI-TOF of BSA (lower trace) and BSA + mPEG (5000 g/mol). In contrast to the polymer-drug conjugate PP7 there is no interaction with BSA.

the paclitaxel molecule has kind of an ''anchor-function''. Other explanations might be found in peculiarities and differences of the ionisation process and corresponding investigations are in progress.

Interactions in the Aqueous Phase

It is well-known from the investigations of Purcell *et al.*[59] and Trynda-Lemiesz [48,449] that paclitaxel interacts with HSA. There has even recently been made use of a receptor-mediated albumin-bound transport mechanism for paclitaxel[9] resulting in a pharmaceutical formulation (ABRAXANE™, American BioScience, Inc.). However, the numbers given in literature concerning the amount of ligands, the equilibrium constants and whether the interaction is specific or unspecific are still under debate [48,49,59].

Since we are interested in the interaction of the prodrug PP7 we have conducted UV- and fluorescence spectroscopical investigations of possible interactions between albumin and PP7. Also preliminary non-denaturating electrophoresis experiments were conducted analogous to those of Purcell [59].

UV-spectrometric investigations showed no isosbestic point but the titration curves indicated that there was a complex formation, which means that there is more than one ligand involved in the interaction of BSA and PP7, Polster and Lachmann [60]. However, since the differences in the spectra were very small it was not possible to determine the exact number of independent absorption equations graphically or with the determinate method, see for example [61]. The results make 2-3 PP7-molecules per BSA-molecule likely with an overall equilibrium constant of 10^4 M^{-1} (order of magnitude) as it was proposed by Purcell [59] for {HSA + x paclitaxel}. This is in accordance with the results of our MALDI-TOF experiments describedabove.

Electrophoretic experiments were unsuccessful in proving any soluble aggregation of albumin and PP7.

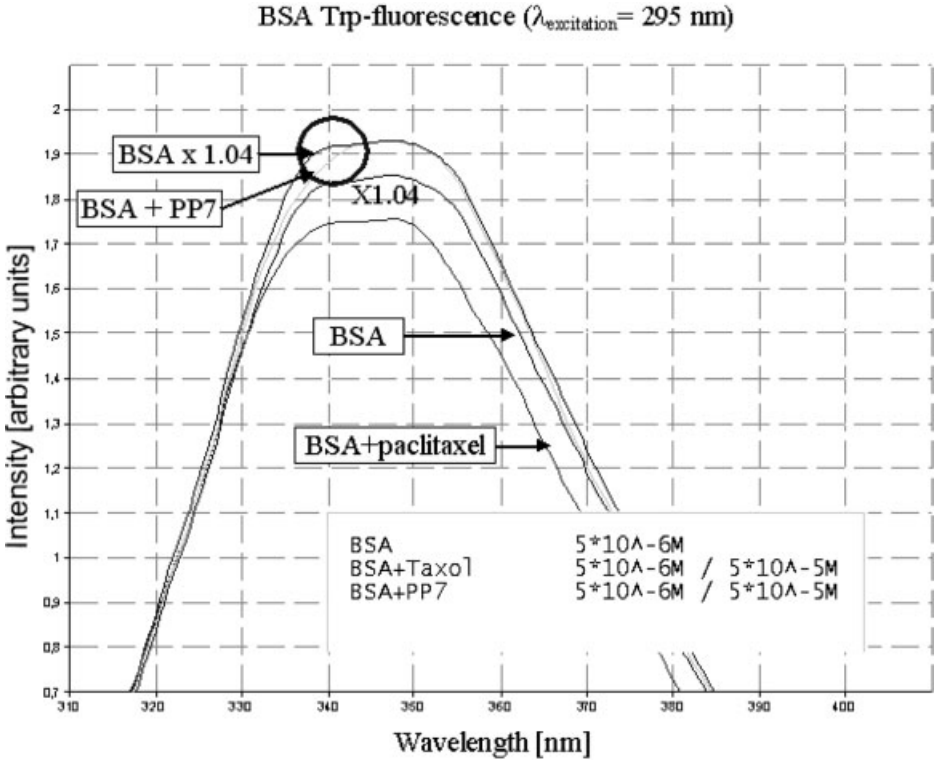


Figure 17.
intensity of the intrinsic fluorescence of BSA trp 214 and trp 134. The pure BSA shows a broad double peak with a shoulder at 340 nm and the main maximum at 347 nm. On addition of paclitaxel (taxol) the fluorescence is quenched. It is known that paclitaxel can enter the hydrophobic cavity near trp 214 but there is more than one docking site, see [48,49,59]. After addition of the mPEG-conjugate of paclitaxel, PP7, the intrinsic fluorescence increases and the short-wave length shoulder disappears, it is probably red-shifted. For comparison the curve of pure BSA is lifted by multiplication with a factor of 1.04 and the missing shoulder can clearly be seen within the circle. It is worth mentioning that the addition of mPEG also leads to an increase of the intrinsic fluorescence in BSA. This is not the case in HSA with only its single trp 214.

Two different types of fluorescence experiments were conducted: there is the intrinsic fluorescence of solutions of trp that shows a maximum at 353 nm in a buffer solution of pH 7. A trp residue in the close to or at the surface of a protein in aqueous solution has usually a fluorescence maximum at 350 nm-353 nm ($\lambda_{ex} = 295$ nm). In general, the fluorescence of proteins originates from trp, phe and tyr. phe has a rather low quantum yield and can be neglected. tyr is efficiently quenched near $-NH_2$, –COOH, and when it is ionized. At $\lambda_{ex} = 295$ 295 nm exclusively trp is excited. If a trp residue is exposed to a more hydrophobic (apolar) environment a blueshift of the emission wavelength is observed. The intrinsic fluorescence of trp is efficiently changed (quenched) when there are major changes of the conformation or trp is near a binding site. However, it also occurs that a ligand forms an even stronger emitting complex with trp, as it is the case with bilirubin and warfarin, for example with trp 214 in HSA and BSA [48,49]. We have used both techniques to investigate possible interactions between albumin and PP7, Fig. 17–20.

The intrinsic fluorescence of (fatty acid free) BSA shows an emission spectrum

($\lambda_{ex} = 295$ nm) with a broad composite peak (340 nm, 347 nm) maximum, Fig. 17. HSA only shows one with a broad unstructured maximum around 340 nm. According to the qualitative rules of fluorescence spectroscopy [62] this means that the 340 nm emission originates from the trp 214 within the hydrophobic pocket of albumin formed by Subdomains IIA and IIIA. The red-shifted maximum at 347 nm would consequently originate from the more surface-exposed trp 134 which is only present in BSA, not in HSA. Since the maximum energy emitted from BSA is about 2.7 times that of HSA, Steinhardt *et al.*[63] concluded that the single HSA-trp is partially quenched while both the BSA-trps are unquenched which is supported by the fact that the fluorescence intensity of the two BSA trps is almost as strong as the fluorescence of two single N-acetyltryptophanamid molecules in solution [64]. It may also be assumed that differences in the fluorescence of BSA and HSA are due to BSA trp 134 which is rather close to the surface but not on the surface. It also appears that trp 134 is much more constraint by the nearby tyrosyl groups. trp 214 on the contrary is buried and protected from polar solvents in a ''very flabby hydrophobic protein matrix'' [65]. However, there is no general agreement of this view in literature and some authors see trp 214 more closer to the polar surface [66–68].

The intrinsic fluorescence of HSA shows a concentration-dependent quenching with paclitaxel that levels out at a rather low concentration while PP7(5000) and mPEG(5000) do not show any effect. BSA also shows a decrease of the fluorescence intensity with the paclitaxel concentration but an increase with PP7, see Fig. 17, and about the same is observed with mPEG.

The composition of the solutions is, a) top to e) bottom:

BSA/warfarin/paclitaxel a) $5 \cdot 10^{-6}$M:$5 \cdot 10^{-6}$M:0; b) $5 \cdot 10^{-6}$M:$5 \cdot 10^{-6}$M:$2 \cdot 10^{-5}$M; c) $5 \cdot 10^{-6}$M:$5 \cdot 10^{-6}$M:$4 \cdot 10^{-5}$M; d) $5 \cdot 10^{-6}$M:$5 \cdot 10^{-6}$M:$6 \cdot 10^{-5}$M; e) $5 \cdot 10^{-6}$M:$5 \cdot 10^{-6}$M:$8 \cdot 10^{-5}$M

The quenching experiments – Fig. 17 – indicate that the hydrophobic pocket of albumin near trp 214 is accessible for paclitaxel but not easily for PP7 with the paclitaxel moiety hidden in the poly(ethylene glycol) coil. However, trp 134 appears to be affected, too. In particular, the intensity of the 347 nm peak grows and the 340 nm peak seems to disappear (see Fig. 17), maybe it is slightly red-shifted due to conformational changes caused by the interaction of albumin with the poly(ethylene glycol) chain that appears to interact with BSA at least near trp 134 with probably an indirect influence on the conformation of subdomain IIA.

As mentioned above there is a strongly fluorescent complex formed with trp 214 and warfarin. Excited at $\lambda_{ex} = 335$ nm this complex shows a broad fluorescence maximum around $\lambda_{em} = 380$ nm, Figs. 18–19.

The quenching of this HSA-fluorescence by paclitaxel alone has already been described by Trynda-Lemiesz [49]. It proves the high affinity of paclitaxel to one of the two major drug-binding sites of albumin at the interface of Sudlow Site I, subdomain IIA, near trp 214.

Our investigations on HSA and BSA confirmed these results and in addition showed that also the presence of PP7 affected the fluorescence of the complex. There is a concentration dependent quenching, which is, however, less efficient compared with the quenching process that is observed in the presence of paclitaxel. At the moment the details of this mechanism are not entirely clear. However, an enzymatic cleavage of PP7 (at least in the case of BSA) with a subsequent action of released paclitaxel appears to be unlikely. What can presently be stated is that there is some interaction between albumin and PP7 that causes a major change of the close environment of trp 214-warfarin complex. The influence of the presence of a ligand on the conformation of a protein was, for example, shown for warfarin-albumin using CD-spectroscopy [69], and also first own CD-experiments have shown an influence of the presence of PP7 on the Cotton-effect of

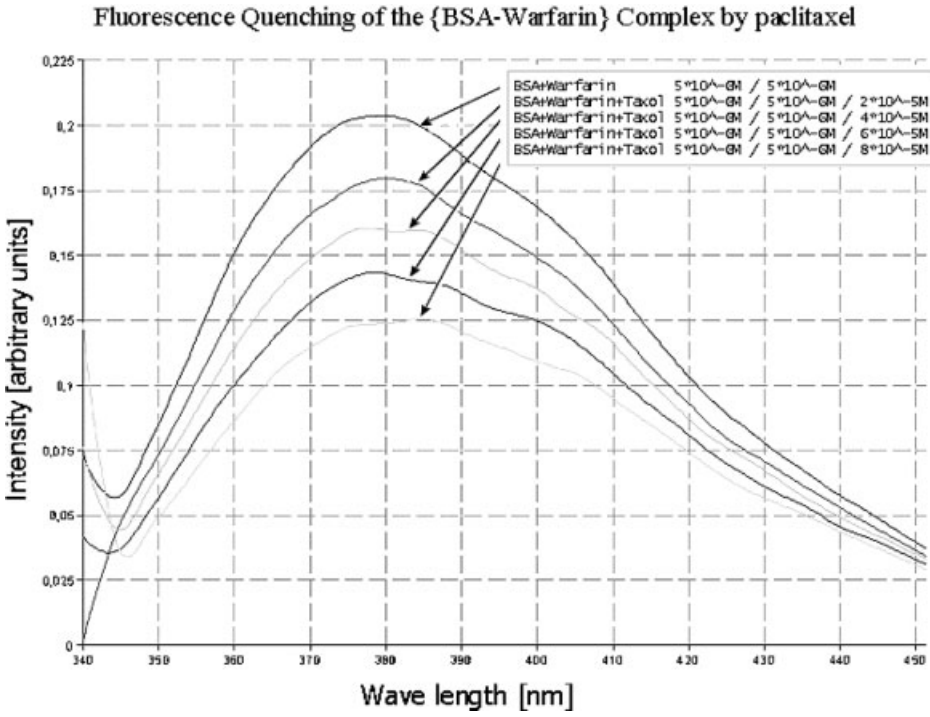


Figure 18.
fluorescence quenching of the emission of the BSA-warfarin complex by different concentrations of paclitaxel (taxol) as indicated in the figure. Paclitaxel does not show a significant fluorescence. The emission peak shows a fine structure.

BSA [70]. Warfarin is not necessarily replaced by the paclitaxel coming with PP7. It can be sufficient to dock PP7 onto a different site with the result of a deformation of the main drug binding cavities in the hydrophobic interior of albumin to cause changes of the fluorescence of the complex. After all, albumine is a highly flexible molecule. However, at the moment a ''threading in'' of the paclitaxel ''head'' of the conjugate into subdomain IIA or at least a docking to its entrance and a deformation of the cavity cannot be excluded although such a process – on the first glance – might not be entropically favoured. On the other hand, the hydrophobic effect might compensate a decrease of the chain conformation of the PEO-chain of PP7. Penetration of PP7 into subdomain IIA would probably require a two-step (second order) mechanism: docking onto albumin and threading of the paclitaxel-end into the hydrophobic pocket.

A Stern-Volmer plot – Fig. 20 – for the quenching of the albumin-warfarin complex fluorescence by paclitaxel and PP7 shows at least in the case of PP7 clearly a combination of static and dynamic quenching since the curve for PP7 is significantly non-linear. The slope of the quenching by PP7 is shallow and indicates a limited access of the quenching species to the fluorescent site. The temperature-dependence of the quenching process can give more information about this process, and corresponding studies are being conducted.

Conclusion

PP7 is an improved water-soluble conjugate of the anti-cancer drug paclitaxel and poly(ethylene glycol). In aqueous solution it does not form molecular aggregates such as micelles or lamellae but shows a molecular solution. The conjugate has the

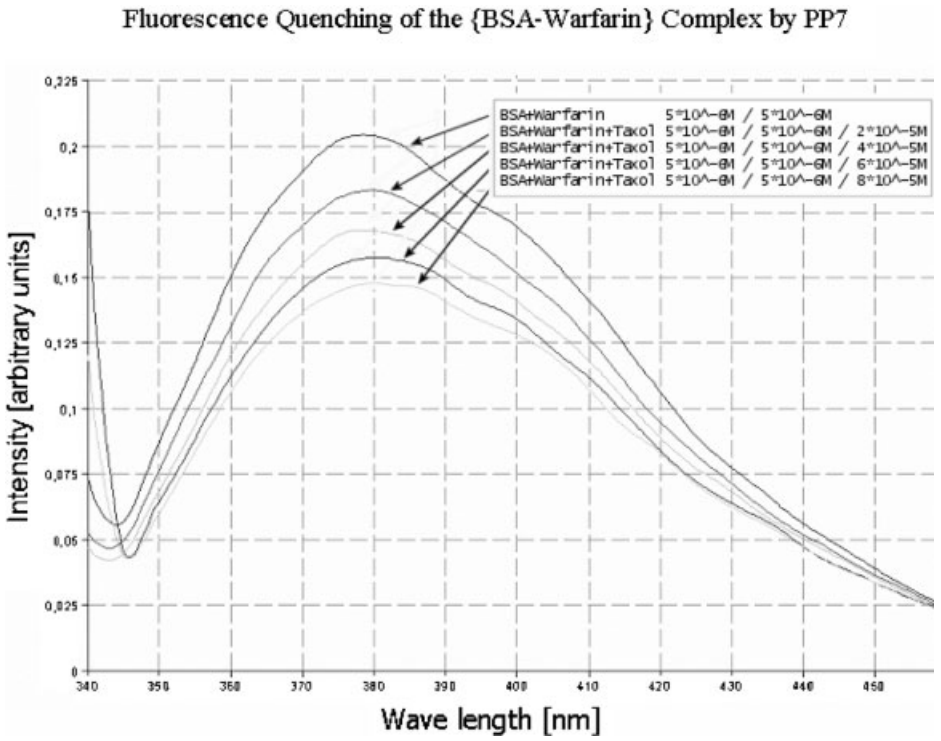


Figure 19.
fluorescence quenching of the BSA-warfarin complex by different concentrations of the paclitaxel-mPEG conjugate PP7 as indicated in the figure and are the same as in Fig. 18. PP7 does not show fluorescence. The emission peak shows a fine structure similar to Fig. 18.

shape of a highly conformational mobile deformed sphere significantly different from the mere polymer coil as could be demonstrated by measurements of the self-diffusion coefficient and the viscosity in dilute solution. The present idea of the molecule is that of a hydrophobic drug surrounded by the hydrophilic

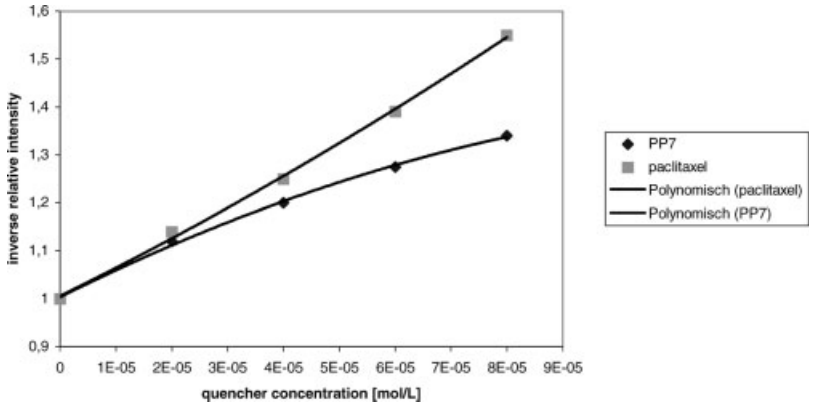


Figure 20.
Stern-Volmer plot of the fluorescence quenching of the BSA-warfarin complex with the pure drug paclitaxel and with the mPEG conjugate PP7. The relative intensity here is defined as intensity of the unquenched fluorescence, I_{fo} intensity divided by the quenched fluorescence intensity, I_{fq}.

poly(ethylene glycol) chain comparable to the conformation of many water-soluble proteins containing a hydrophobic core.

Complex formation of paclitaxel with human serum albumin (HSA) and bovine serum albumin (BSA) are known, now also the interaction of a polymer-drug conjugate (PP7) with albumin could be shown. No interaction of the mere poly(ethylene glycol) with BSA – neither in the solid state nor in solution – was found. Apparently, paclitaxel shows some kind of an ''anchor-effect'' that allows the drug-polymer conjugate to dock onto albumin with subsequent effects on the conformation of the PEO-chain and the protein.

The competition of PP7 with other ligands of Sudlow Site I is weak compared with paclitaxel. This is important in combined therapies but also for the transport mechanism of paclitaxel respectively PP7. A paclitaxel conjugate that aggregates to larger (nano-sized) particles should clearly have advantages in drug delivery through its higher local concentration, longer circulation time and the possibility to tailor size and properties to accumulate in tumour tissue and set its cytostatic potential predominantly free where it is required.

While the drug moiety of PP7 is not able to penetrate completely into one of the primary drug-binding sites of albumin it is at least able to change significantly the environment of the strongly fluorescent albumin-warfarin complex. Comparable effects can be assumed for other albumin complexes.

Complexes with at least 3 PP7 ligands to albumin were determined in the solid state, and they are still stable in the vapour phase of a MALDI-TOF experiment.

These experimental results give rise to further investigations of modified paclitaxel-polymer conjugates with enhanced capability of the formation of supramolecular complexes by self-aggregation and/or to control complex formation with serum constituents and interaction with membrane proteins. Further improvement of a targeted drug delivery may be possible by size control of the aggregates and making use of the super-permeability of tumour tissue, hence opening some more ways to fight different cancer types individually.

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